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QUANTITATIVE ANALYSIS OF SURVIVIN mRNA EXPRESSION IN BLADDER TRANSITIONAL CELL CARCINOMAS

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We assessed the expression levels of survivin mRNA in bladder transitional cell carcinoma (TCC) to provide additional information regarding its malignant potential. The real-time PCR method was used to detect the survivin mRNA level for 21 bladder tumor specimens, and for urinary exfoliated cells from 12 newly diagnosed bladder tumor patients. All bladder tumor specimens and 7 of 12 voided urine specimens expressed survivin mRNA. In tumor specimens, high grade, high stage tumors had the tendency to express more survivin mRNA. Of 12 superficial bladder tumor patients who had transurethral resection of bladder tumor (TURB), 3 showed high survivin mRNA expression and intravesical recurrence after the surgery. However, for the patients who had total cystectomy due to invasive tumor, no relations were observed between the survivin mRNA expression level and development of local recurrence and/or distant metastasis. Our results suggested that the quantitative analysis of the survivin mRNA may indicate local malignant potential, which contribute to the possibility of an intravesical recurrence.

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Key words : Survivin, Bladder neoplasms, Urine

INTRODUCTION

Survivin, a novel and structurally unique member of the inhibitor of apoptosis (IAP) gene family, suppresses apoptosis in combination with Caspase 3, Caspase 7 by means of a baculoviral IAP repeat (BIR)[1,2]. A characteristic finding is that survivin is expressed during embryonic and fetal development, and then becomes completely down-regulated and undetectable in normal adult tissues, however, it becomes prominently expressed in transformed cell lines and in most of the common human cancers such as the lung, colon, pancreas, prostate and breast[1]. The common pathway of apoptosis is the activation of Caspase 3, Caspase 7 or Caspase 6, hence, the high expression of survivin may protect cells from many apoptosis signals[3,4]. The widespread survivin overexpression in many types of cancers might indicate that survivin plays a major role in carcinogenesis[5–7].

Several studies have shown that the high survivin expression correlates with a poor prognosis and chemotherapy resistance[8,9]. In bladder cancer, Swana et al. reported that survivin expression correlated with decreased time to the intravesical recurrence of transitional cell carcinoma using immunohistochemical methods[10]. Recently, Weikert et al.[11] reported quantitative analysis of survivin mRNA expression in urine and bladder tumor tissue concerning its potential relevance for disease prognosis. In this study, we also investigated the expression of survivin mRNA in bladder transitional cell carcinoma by our own way and obtained the supportive results to them.

MATERIALS AND METHODS

Patient population

Tumor tissue specimens and/or voided urine samples were collected from 21 patients with bladder cancer and from 4 patients with benign genitourinary disease ; with median age was 70.3 and 60 years respectively. The characteristics of patients with bladder cancer are shown in Table 1. This study was approved by the ethics committee of the Tokyo Medical University Hachioji Medical Center, and all patients gave us their written informed consent.

Sample collection

Tumor tissue samples were obtained from 13 cases by transurethral resection of bladder tumor (TURB) and 8 cases by cystectomy. Four normal bladder mucosa specimens were also obtained from patients who underwent surgery for benign prostatic hyperplasia (n=3) and urolithiasis (n=1). All tissue specimens were snap-frozen immediately after collection and stored at −80°C. Spontaneously voided urine specimens were also obtained from 12 of 13 patients with newly diagnosed bladder tumor before treatment. The urine specimens were examined by survivin mRNA measurement assay and urinary cytology. We considered the urinary cytology findings to be positive when the results showed class IV–V. The urine specimen for the survivin assay was cooled on ice immediately, and centrifuged. The pellets were stored at −80°C until quantitative analysis.

Quantitative analysis of survivin mRNA by real-time

Table 1. Summary of the clinical findings and survivin mRNA levels in bladder transitional cell cancer tissue specimens

Case No.	Age/sex	Diagnosis	Grade	Stage	Multiplicity	Size	Survivin mRNA levels	Bladder status
1	73/M	Primary	2	pT3b	Multiple	Large	5.26	TC
2	81/M	Primary	1	pTa	Single	Large	4.79	NED
3	71/M	Primary	2	pTa	Single	Large	5.42	TC
4	73/M	Primary	3	pT3b	Single	Large	5.35	TC
5	73/F	Primary	3	pT1	Single	Small	5.43	Rec.
6	45/M	Primary	3	pT1	Single	Large	5.76	Rec.
7	64/M	Primary	1	pTa	Single	Small	5.17	NED
8	54/M	Primary	3	pT2a	Multiple	Large	5.57	TC
9	61/M	Primary	2	pT4	Multiple	Large	4.94	TC
10	63/M	Primary	3	pT1	Multiple	Large	5.84	Rec.
11	62/M	Primary	3	pT2	Multiple	Large	6.48	TC
12	74/M	Primary	3	pT2	Multiple	Large	5.87	NED
13	70/F	Primary	3	pT3	Single	Large	5.02	TC
14	39/M	Recurrent	1	pTa	Multiple	Small	5.12	NED
15	58/M	Recurrent	1	pTa	Multiple	Small	5.07	NED
16	58/M	Recurrent	3	pT2a	Single	Small	5.84	TC
17	71/F	Recurrent	1	pTa	Multiple	Small	4.88	NED
18	70/F	Recurrent	1	pTa	Multiple	Small	4.24	NED
19	69/M	Recurrent	1	pTa	Single	Small	4.77	NED
20	85/M	Recurrent	2	pTa	Multiple	Large	4.8	NED
21	76/M	Recurrent	1	pT1	Single	Small	5.26	NED

Multiplicity : Endoscopic findings. Multiple, multiple tumors. Single, single tumor. Size : Main tumor size. Large, 1 cm or more. Small, less than 1 cm. Survivin levels : survivin mRNA level is expressed as log copies/μg total RNA. Bladder status ; Status of urinary bladder at approximately 6 months after treatment. TC, after total cystectomy. NED, no evidence of disease. rec., intravesical recurrence.

PCR

All tissue samples were enrolled for survivin mRNA detection using the real-time PCR method. The expression intensity of the survivin gene was calculated as a relative value based on the standard RNA, and it was rearranged according to the intensity. To prepare standard RNA for quantification, the PCR product was cloned into a pBluescript vector (STRATAGEN, CA, USA) and then was linearized to prevent any activity at the T3 promoter site. Standard RNA was synthesized at 37°C/1 hour with T7 RNA polymerase (Invitrogen, CA, USA). After incubation, Synthesized RNA was purified by RNAzol and DNase I (TaKaRa, Shiga, Japan) treatment.

Total RNA was extracted from the frozen sections by the acid guanidinim thiocyanate-phenol-chloroform extraction method. cDNA was synthesized with a survivin Reverse primer (SVN-R) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), as an internal control, exon 4 primer (5′-CGG TGC CAT GGA ATT TGC CC-3′). The PCR reaction mixture was prepared using a TaqMan universal Master Mix (PE Applied Biosystems, CA, USA). The primer set to amplify survivin mRNA was designed according to GenBank NM001 168, using primers exon 1 (SVN-F) : 5′-AGA ACT GGC CCT TCT TGG AGG-3′ and exon 2－3 (SVN-R) : 5′-CTT TT TAT GTT CCT CTA TGG GGT C-3′. The probe exon 1－2 (SUV-P) : 5′-AGC GGA TGG CCG AGG CTG GCT TC-3′ was designed to target the internal region between the SVN-F and SVF-R primers. The primer/probe set for the amplification of GAPDH mRNA was Human GAPDH primer/probe mix (PE Applied Biosystems, CA, USA). Each PCR reaction was made at 50 cycles (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds) using a real-time PCR system (ABI PRISM 7700 Sequence Detection System : PE Applied Biosystem). GAPDH mRNA was used for real-time PCR as an internal control.

The PCR products of survivin mRNA were purified using a highly pure PCR product purification kit (Roche Molecular Biochemicals Diagnostic, Inc., USA) and were directly sequenced using the BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems). The sequence was finally compared with each target mRNA sequence.

Statistical analysis

The associations between the clinico-pathological factors and the survivin mRNA expression levels were examined using nonparametric Mann-Whitney U-test. Multivariate analysis was performed by multiple regression analysis. Correlations between survivin mRNA level and cytology in urinary exfoliated cells were

analyzed using chi-square test. The significance level was established at 0.05 for all statistical tests.

RESULTS

Survivin mRNA levels in bladder tumors

We quantified the expression of survivin mRNA in 21 TCC with varying stage and grade categories and 4 normal bladder mucosa specimens. A RT-PCR analysis revealed sufficient GAPDH expression in all samples investigated. We demonstrated the expression of survivin mRNA in all tumor specimens, but the survivin mRNA expression was not detected in any of the normal bladder mucosa specimens. The survivin mRNA levels in TCC specimens are shown in Table 1. In addition, the statistical differences between the clinico-pathological factors of bladder tumors and the survivin mRNA levels are shown in Table 2. The survivin mRNA levels in high-grade tumors were significantly higher than in those with low-grade tumors ($P=0.001$). For stage, the survivin mRNA levels in superficial tumors (Ta/T1) significantly differed from the muscle-invasive tumors (T2/T3/T4) ($P=0.038$). The survivin mRNA levels in primary tumors were statistically higher than in recurrent tumors ($P=0.019$). Of those, only tumor grade indicated the statistical correlation to the survivin expression level by multivariate analysis (grade ; $P=0.007$, stage ; $P=0.830$, diagnosis ; $P=0.507$).

Correlation between the survivin expression levels and patient outcome

The survivin mRNA expression level with regard to the clinical outcome of all patients were also evaluated. Of 13 superficial Ta/T1 tumors, 1 case (Case No. 3, Table 1) underwent total cystectomy because the tumor size was too large. The remaining 12 cases had conventional TURB. Eight of 12 cases received adjuvant intravesical instillation therapy, with Bacillus Calmette-Guérin (BCG) (Cases No. 5, 6, 10, and 20, Table 1) or epirubicin hydrochloride (Cases Nos. 14, 17, 18, and 19). Among the superficial bladder tumor cases treated with TURB, 3 of 12 (Cases No. 5, 6, 10) showed intravesical recurrence. The survivin mRNA levels of these 3 cases ranged from $10^{5.43}$–$10^{5.84}$ copies/μg total RNA. These were significantly higher than those of the non- local recurrence patients ($10^{4.79}$–$10^{5.26}$ copies/μg total RNA) ($P=0.016$).

All the cases having invasive tumors (T2–T4 tumor stage) received total cystectomy except for 1 case (Case No. 12, Table 1). Five cases (Cases No. 1, 4, 8, 9 and 13) received adjuvant systemic chemotherapy. One case (Case No. 1) had local recurrence and another 2 cases (Cases No. 4 and 13) had distant metastases within 6 months after being treated. Survivin mRNA levels of the tumors in these 3 cases (Cases No. 1, 4 and 13) with local recurrence or metastases were not significantly different from those in the other 4 cases (Cases No. 8, 9, 11 and 16) showing no recurrence or metastasis after total cystectomy ($P=0.376$).

Correlation between urinary cytology and survivin mRNA expression

We examined the survivin mRNA levels of the urinary exfoliated cells from primary bladder TCC patients and from 4 patients with benign genitourinary disease before treatment. Seven of 12 patients (58.3%) with primary

Table 2. Correlation between the clinico-pathological factors and the survivin mRNA levels

Clinico-pathological factors	n	Survivin mRNA level			p value
		Mean	Range	Median	
Diagnosis					
Primary	13	5.45	4.79–6.48	5.42	0.019
Recurrent	8	4.99	4.24–5.84	4.98	
Grade					
Low grade	12	4.98	4.24–5.42	5.01	0.001
High grade	9	5.68	5.02–6.48	5.76	
Stage					
Superficial	13	5.12	4.24–5.84	5.12	0.038
Invasive	8	5.54	4.94–6.48	5.46	
Multiplicity					
Single	11	5.28	4.77–5.84	5.31	N.S.
Multiple	10	5.28	4.24–6.48	5.12	
Size					
Small	9	5.09	4.24–5.84	5.12	N.S.
Large	12	5.43	4.79–6.48	5.38	

The survivin mRNA level is expressed as log copies/μg total RNA. p value is analyzed by the Mann-Whitney U-test. N.S., statistically not significant. Grade: Low grade, G1 or G2. High grade, G3. Stage: Superficial, pTa or pT1. Invasive, pT2 or more.

Table 3. Correlation between urinary cytology and survivin mRNA expression

Urinary Cytology	Urinary survivin mRNA expression		Total
	Positive	Negative	
Positive (IV–V)	4	2	6
Negative (I–III)	3	3	6
Total	7	5	12

bladder TCC had detectable urinary survivin mRNA levels, ranging from $10^{2.47}$ to $10^{6.14}$ copies/μg total RNA, but none of the urine samples from benign cases showed detectable survivin mRNA. Although we did not detect any survivin mRNA expression in urinary exfoliated cells obtained from 5 TCC patients, the tumors themselves did express survivin mRNA. The correlation between urinary cytology and survivin mRNA expression are shown in Table 3. The sensitivity of urinary survivin assay and cytology for detecting bladder cancer was not statistically different ($P=0.558$).

DISCUSSION

Survivin is unique among IAP proteins due to its sharp differential expression in a series of human cancers, and it is widely accepted that survivin is closely related to the onset and development of cancer. A high incidence of survivin expression in bladder cancer was previously determined by immunohistochemistry[10,12] and RT-PCR assays[11,13]. In the present study, survivin mRNA was detected in 21 of 21 (100%) cases of TCC tissues and in none of 4 cases of normal bladder mucosa. These data suggest that the high expression of survivin is a common phenomenon in TCC and the inhibition of apoptosis by survivin expression may thus play an important role in property of malignant cells.

This above phenomenon supports the hypothesis that survivin participates in tumor progression rather than in oncogenic transformation[14]. A correlation between the survivin expression with aggressive tumor behavior and a poor prognosis has been demonstrated for colorectal carcinoma[15] and breast cancer[16]. In TCC, the relationship between the survivin expression and clinical outcome has also been reported in previous studies[11–13]. Whether the survivin expression is related to the grade and stage of superficial bladder cancers is still debatable[10,12]. Although the number of patients in our study were limited, significant higher levels of survivin mRNA expression were found in high-grade tumors and invasive tumors ($P=0.001$, $P=0.038$). By multivariate analysis, only the tumor grade category was statistically associated with expression levels of survivin mRNA. Weikert et al.[11] who used RT-PCR to examine 53 TCC cases of the urinary bladder, reported that the levels of survivin mRNA were correlated with pathological grade and stage. These findings indicate that survivin may influence the malignant potential of transformed transitional cells, thereby playing a role in the progression of bladder cancer.

Bladder cancer is a malignant tumor, and even when identified at an early stage, many patients develop cancer recurrence. If survivin gene expression is a useful marker for the malignant potential of bladder cancer, then the therapeutic methods could be changed according to the expression levels of the survivin gene. According to our clinical data, in patients with superficial tumor, the expression level of survivin mRNA was correlated with local recurrence after TURB ($P=0.016$). Although recurrent cases were all grade 3 tumors in the present study, Weikert et al.[11] also reported that the higher levels of survivin mRNA expression were associated with reduced time to recurrence in superficial bladder tumors. Swana et al.[10], who used immunohistochemistry, also reported that the time to first recurrence was significantly shorter in patients with survivin positive grade 1 tumors than in those with survivin negative grade1 tumors. These reports supported our findings. On the other hand, the expression level of survivin mRNA in our patients with recurrent tumor was significantly lower than that of newly diagnosed primary tumors. However, this contradictory result may have been caused by the fewer high grade tumors in the recurrent tumor group compared to the primary tumor group.

In our patients with invasive bladder tumor who were treated by total cystectomy, the survivin mRNA levels were not correlated with the development of local recurrence or distant metastases ($P=0.376$). Weikert et al.[11] also reported that the time to metastasis or relapse was not related with the expression levels of survivin mRNA in patients with invasive bladder tumor. Considering these facts, the expression levels of survivin mRNA may only contribute to an intravesical tumor behavior.

We also analyzed the gene expression of survivin mRNA in naturally voided urinary exfoliated cells from patients with bladder tumor. In our study, although all tumors expressed survivin mRNA, no correlation could be found between the tissue and urinary survivin mRNA levels. Regardless of whether survivin detection strategies in urine samples are based on protein or mRNA analyses, they should yield comparable results. Since survivin is a short-lived nonsecreted protein[14], its detection depends on the abundance of exfoliated malignant cells.

Our findings that the tissue survivin expression levels were correlated with pathological features and intravesical recurrence in patients with noninvasive bladder cancer tumors consequently supported the recent report[11]. As a quantitative marker, survivin may provide some more information for the current intensive follow-up protocols to bladder cancer, though further studies and large series of cases should be added to validate the prognostic value of survivin analysis.

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REFERENCES

1) Ambrosini G, Adida C and Altieri DC : A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med **3** : 917–921, 1997
2) Johnson AL, Langer JS and Bridgham JT : Survivin as a cell cycle-related and antiapoptotic protein in granulosa cells. Endocrinology **143** : 3405–3413, 2002
3) Tamm I, Wang Y, Sausville E, et al. : IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res **58** : 5315–5320, 1998
4) Conway EM, Pollefeyt S, Steiner-Mosonyi M, et al. : Deficiency of survivin in transgenic mice exacerbates Fas-induced apoptosis via mitochondrial pathways. Gastroenterology **123** : 619–631, 2002
5) Xu AG, Li SG, Liu JH, et al. : Function of apoptosis and expression of the proteins Bcl-2, p53 and C-myc in the development of gastric cancer. World J Gastroentero **7** : 403–406, 2001
6) Shan CM and Li J : Study of apoptosis in human liver cancers. World J Gastroentero **8** : 247–252, 2002
7) Zhang Z, Yuan Y, Gao H, et al. : Apoptosis, proliferation and p53 gene expression of H. pylori associated gastric epithelial lesions. World J Gastroentero **7** : 779–782, 2001
8) Ikeguchi M and Kaibara N : Survivin messenger RNA expression is a good prognostic biomarker for oesophageal carcinoma. Br J Cancer **87** : 883–887, 2002
9) Chakravarti A, Noll E, Black PM, et al. : Quantitatively determined survivin expression levels are of prognostic value in human gliomas. J Clin Oncol **20** : 1063–1068, 2002
10) Swana HS, Grossman D, Anthony JN, et al. : Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. N Engl J Med **341** : 452–453, 1999
11) Weikert S, Christoph F, Schrader M, et al. : Quantitative analysis of survivin mRNA expression in urine and tumor tissue of bladder cancer patients and its potential relevance for disease detection and prognosis. Int J Cancer **116** : 100–104, 2005
12) Ku JH, Kwak C, Lee HS, et al. : Expression of survivin, a novel inhibitor of apoptosis, in superficial transitional cell carcinoma of the bladder. J Urol **171** : 631–635, 2004
13) Schultz IJ, Kiemeney LA, Witjes JA, et al. : Survivin mRNA expression is elevated in malignant urothelial cell carcinomas and predicts time to recurrence. Anticancer Res **23** : 3327–3331, 2003
14) Altieri DC : Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene **22** : 8581–8589, 2003
15) Williams NS, Gaynor RB, Scoggin S, et al. : Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. Clin Cancer Res **9** : 931–946, 2003
16) Tanaka K, Iwamoto S, Gon G, et al. : Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res **6** : 127–134, 2000

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和文抄録

膀胱移行上皮癌患者における半定量的サバイビン mRNA 測定法の検討

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膀胱移行上皮癌患者におけるサバイビン mRNA 発現の診断・予後因子としての有用性を検討した．リアルタイム PCR 法を用い膀胱移行上皮癌患者21例の膀胱癌組織と初発例12例の自排尿中のサバイビン mRNA 発現量を半定量的に測定し，すべての腫瘍組織と12例中 7 例の自排尿中にサバイビン mRNA の発現を認めた．サバイビン mRNA の発現量は低分化癌および浸潤性腫瘍で有意に高値であった．表在性膀胱癌症例のうちサバイビン mRNA の発現量が高い 3 例で再発を認めた．浸潤性膀胱癌症例ではサバイビン mRNA の発現量と局所再発あるいは遠隔転移とは関係がなかった．これらのことより膀胱移行上皮癌患者におけるサバイビン mRNA の半定量的測定は膀胱内再発についての悪性度を示している可能性が考えられた．

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